Survival enhancement of mesenchymal stem cells by phosphoinositide 3-kinase after transplantation into the infarcted myocardium

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Directed by Professor Jun Hee Sul

The Doctoral Dissertation submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

Survival enhancement of mesenchymal stem cells by phosphoinositide 3-kinase after transplantation into the infarcted myocardium

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Mesenchymal stem cell (MSC) therapy for myocardial regeneration has a limitation due to the poor survival of stem cell after cell transplantation in infarcted heart. Members of the phosphoinositide 3-kinase (PI3K) family regulate fundamental cellular responses, including proliferation, growth, chemotaxis, and survival. To improve the cell survival in the infarcted heart, MSCs were genetically engineered to overexpress class II PI3K α (PI3K-C2α).

Expression of endogenous PI3K-C2α was time-dependently decreased in hypoxic condition. PI3K-C2α overexpression exerted an increase of endogenous PI3K-C2α expression and of cell viability in MSCs. The survival signaling, including phosphorylation of Akt, Bad, cAMP-response element-binding protein (CREB) and IκB kinase (IKK) respectively augmented in PI3K-C2α-MSCs. The Bcl-2/Bax ratio of PI3K-C2α-MSCs also had a great increase under hypoxic condition for 12 hr. However, the cleavage of poly (ADP-ribose) polymerase (PARP) expression and PI positive cells were decreased in PI3K-C2α-MSCs compared to hypoxic MSCs. The adhesion of MSCs was strengthened by overexpressing of PI3K-C2α on cardiogel (3D-matrix). Nine rats per group were transplanted with $1x10^6$ cells (20 μl PBS) after myocardial infarction. One week after transplantation, reduced infarct size and fibrosis area were observed in PI3K-C2α-MSCs-transplanted group. According to morphologic analysis, TUNEL positive cells and neutrophils were also declined, but the mean microvessel count per field was higher in PI3K-C2α-MSCs group compared to MSCs-injected group. These findings suggest that overexpression of PI3K-C2α in MSCs can assist cell survival and enhance myocardial regeneration.

Key words: mesenchymal stem cells, PI3K-C2α, survival, lentivirus, cardiac regeneration

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I. INTRODUCTION

Acute or chronic myocardial ischemia causes cellular damage or death with fibrotic scar formation, physiological dysfunction, electrical uncoupling, and loss of structural integrity, all of which may lead to remodeling of the left ventricle (LV).[1] Attempts have been made to restore myocardial infarction using several different cell types to determine the best cell type for cardiac regeneration.[2] Among the various stem cell types, mesenchymal stem cells

(MSCs) appear to be the most promising cell type for repairing an ischemic myocardium. MSCs are a special stromal population of multipotent cells that can differentiate into cardiac myocytes, endothelial cells, neural cells, or cells of the endoderm, both in vitro and in vivo, given the appropriate conditions.[3-5] Although MSC therapy can improve LV function and the contractile performance of an infarcted heart, poor cell survival due to the harsh conditions present at the transplantation site remains a major clinical problem.[6-9]

To overcome the poor survival of MSCs in infarcted myocardium, the molecular mechanism of cell apoptosis and necrosis needs to be elucidated to improve stem cell survival.[10] Factors that cause cell death in this context include the ischemic conditions, mechanical stress, the host inflammatory response, and proapoptotic factors.[11,12] Induction of anti-apoptotic proteins or blocking of caspases and a cytoprotective state (heat shock or induction of a hypoxia response) may enhance the survival of MSCs under ischemic conditions. Phosphoinositide 3-kinases (PI3K)/Akt signaling is known to regulate survival signaling.

The activation of phosphoinositide 3-kinases (PI3K) in response to extracellular stimuli is a hallmark of many signaling pathways.[13,14] This signaling pathway can be classified into three groups according to their primary structure and regulation (Table 1).[15,16] The major studies focused on class I and class III PI3Ks, which generate PtdIns-3,4,5-trisphosphate and PtdIns-3-monophosphate,

respectively. Each class plays important role in growth factor signaling and amino acid sensing, and are uncontrolled in disease states, such as cancer and diabetes.[17] There are fewer reports that the role of class II PI3Ks is studied in cellular signaling and disease. The 3 enzymes (C2α, C2β, and C2γ) are large molecules ranging about 166 to 190 kDa. PI3K-C2α and PI3K-C2β are widely expressed on the mRNA level, whereas PI3K-C2γ is expressed in liver, breast and prostate only.[18] In contrast to classes I and III PI3Ks, which are both dimeric enzymes, class II PI3Ks are monomeric enzymes with extended NH2 and COOH termini. These NH2 and COOH termini include multiple protein and lipid interaction domains that target them to various intracellular membranes and the nucleus.[19,20]

Table 1. Three classes of PI3Ks

		subunits
Class	Catalytic	Adaptor
I A	p110α,β,δ	p85α, p55α, p50α, p85β, p55γ
IB	p110γ	p101, p84
II	PI3K-C2α,β,γ	?
III	Vps34p analogues	p150

This study was hypothesized that MSCs that were genetically modified to have an increased survival rate would have improved viability after stem cell transplantation compared to non-genetically modified MSCs. To prevent the death of MSCs under hypoxic condition, lentivirus-PI3K was transfected into MSCs and it was analyzed survival/apoptotic signaling marker proteins. Furthermore, MSCs overexpressing PI3K were implanted into infarcted rat hearts, and examined and compared the physiological and morphological changes in these hearts compared to infarcted hearts transfected with non-modified MSCs.

II. MATERIALS AND METHODS

1. Isolation and culture of MSCs

MSC isolation and characterization were performed as described previously.[21,22] MSCs were isolated from the femoral and tibial bones of rats. Bone marrow-derived MSCs were collected from the aspirates of the femurs and tibias of 4-week-old male Sprague-Dawley rats (approximately 100 g) with 10 ml of MSC medium consisting of Dulbecco's modified Eagle's medium (DMEM)-low glucose, supplemented with 10% fetal bovine serum and 1% antibiotic-penicillin and streptomycin solution. Mononuclear cells recovered from the interface after centrifugation in Ficoll (GE healthcare) were washed twice, resuspended in 10% fetal bovine serum (FBS)-DMEM, and plated in flasks at 1×10^6 cells per 100 cm^2. Cultures were maintained at 37℃ in a humidified atmosphere containing 5% CO_2. After 48 or 72 hr, nonadherent cells were discarded, and the adherent cells were thoroughly washed twice with phosphate-buffered saline (PBS). Fresh complete medium was added and replaced every 3 or 4 days for approximately 10 days. All MSCs were used passage 4. The characteristics of MSCs were demonstrated by immunophenotyping. To verify the nature of cultured MSCs, cells were labeled for various surface and intracellular markers, and analyzed by flow cytometry. Cells were harvested, washed with PBS, and labeled with antibodies against

CD14, CD34, CD71, CD90 or CD105 conjugated with fluorescein isothiocyanate (FITC) or Texas red. FITC-conjugated goat anti-mouse IgG and Texas red-conjugated goat anti-rabbit IgG from Jackson Immunoresearch Immunoresearch Laboratories were used as secondary antibodies. Labeled cells were assayed by flow cytometry and analyzed with Cell-Quest Pro Software (Becton Dickinson).

2. Lentiviral transduction of MSCs

For stable genetic modification, we used the lentiviral vector (LentiV) for transgene delivery. First passage MSCs were prepared for infection by PI3K-C2α-overexpressing lentivirus, hCMV-Pik3c2a/eGFP IRES puro (LentiH1.4-Pik3c2a/eGFP, Macrogen). MSCs were transduced by adding purified Lentivirus to cells with 7 μg/ml polybrene to facilitate transduction followed by incubation for at least 12 hr. GFP-labeled MSC colonies were microsurgically segregated from non-green cells, followed by culturing under nondifferentiating conditions for expansion. This process was repeated until a homogenous population of green MSCs was obtained (Fig. 1).

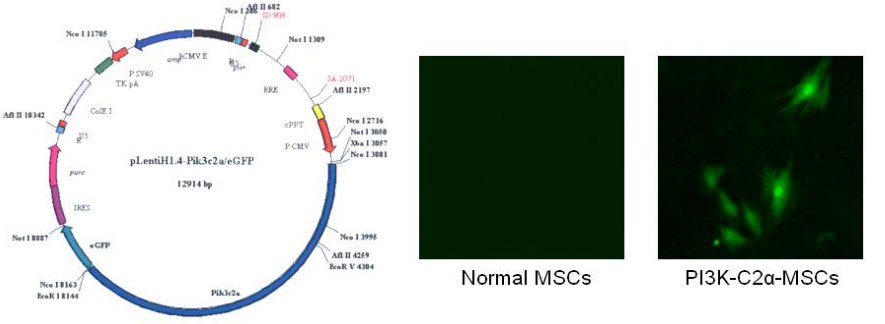

Figure 1. Construction and transduction activity of PI3K-C2α-lentiviral vector in MSCs. Left panel is the structure of PI3K-C2α-Lentiviral vectors. Right panel is the immunofluorescence that expression levels of cellular PI3K-C2α were determined by GFP.

3. Immunoblot analysis

Cells were washed once in PBS and lysed in a lysis buffer (Cell Signaling Technology) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na2-EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined using a Bradford Protein Assay Kit (Bio-Rad). Proteins were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore). After blocking the membrane with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 10% nonfat dried milk for 1 hr at room temperature, the membrane was washed twice with TBS-T and incubated with primary antibody for 1 hr at room temperature or overnight at 4℃. The membrane was washed three times with TBS-T for 10 min and then incubated for 1 hr at room temperature with horseradish peroxidase-conjugated secondary antibodies. After extensive washing, the bands were detected by enhanced chemiluminescence reagent (Santa Cruz Biotechnology). The band intensities were quantified using the Photo-Image System (Molecular Dynamics).

4. Cell viability assay

Cellular survival rate was measured with the PreMix WST-1 Cell Proliferation

Assay System (Takara Bio). Cells (2×10^4) were seeded into wells of a 96-well culture plate and incubated under hypoxic conditions after transduction of lentiviral vector. WST-1 cell proliferation reagent was added directly to the supernatant (10 μl/100 μl growth medium), and incubated at 37℃ for 2 hr. The absorbance of the solubilized dark red formazan product was then determined at 450 nm.

5. Flow cytometric analysis

To evaluate rates of cell transduction and cell death, culture medium was removed from the PI3K-MSCs and the adherent cells were trypsinized and resuspended. These suspensions were then added to their respective culture medium. After centrifugation at 1500 rpm for 5 min, cell pellets were resuspended in PBS containing propidium iodide (PI) (final concentration 1 μg/ml). Samples were analyzed in a FACSCalibur (Becton Dickinson Biosciences) flow cytometer to score PI-positive and PI-negative GFP positive cells.

6. Preparation of cardiac fibroblast-derived three-dimensional matrix (Cardiogel)

Cardiogel was prepared with a minor modification. Briefly, 2×10^5 cells per 35-mm dish were seeded and the medium changed every 48 hr until the matrix

was denuded of cells. The medium was carefully aspirated and rinsed gently with PBS. Next, 1 ml of prewarmed extraction buffer (0.5% Triton X-100, 20 mM NH_4OH in PBS) was added, and the process of cell lysis was observed using an inverted microscope until no intact cells were visualized. The cellular debris was washed with PBS, and the matrices were incubated at 37℃ for 30 min with 1 ml of DNase (10 units of DNase per milliliter of PBS) to minimize the DNA debris. The matrix-coated plates were covered with a minimum of 3 ml of PBS containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone.

7. Induction of myocardial infarction and transplantation

All experimental procedures for animal studies were approved by the Committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine, and performed in accordance with the Committee's Guidelines and Regulations for Animal Care. Myocardial infarction was produced in male Sprague-Dawley rats (250 ± 30 g) by surgical occlusion of the left anterior descending coronary artery, according to previously described procedures. Briefly, after induction of anesthesia with ketamine (10 mg/kg) and xylazine (5 mg/kg), the third and fourth ribs were cut to open the chest, and the heart was exteriorized through the intercostal space. The left coronary artery was ligated 2–3 mm from its origin with a 5-0 prolene suture (ETHICON) for 3

days. Ligature ends were passed through a small length of plastic tube to form a snare. For coronary artery occlusion, the snare was pressed onto the surface of the heart directly above the coronary artery and a hemostat applied to the snare. Ischemia was confirmed by the blanching of the myocardium and dyskinesis of the ischemic region. After 60 min of occlusion, the hemostat was removed and the snare released for reperfusion, with the ligature left loose on the surface of the heart. Then stem cells were injected into the border zone. For transplantation, cells were suspended in 10 μl of serum-free medium (1×10^6 cells) and injected from the injured region to the border using a Hamilton syringe (Hamilton Co.) with a 30-gauge needle. Throughout the operation, animals were ventilated with 95% O_2 and 5% CO_2 using a Harvard ventilator. Operative mortality was 10% within 48 hr. There are three groups of nine rats each used in this study as follows: Sham, ligated but not implanted rat; MSC, ligated and MSCs implanted rat; PI3K-MSC, ligated and PI3K-C2α-transduced MSCs implanted rat.

8. Determination of infarct size

TTC staining was used to assess myocardial tissue viability and determine myocardial infarct size. The tissue slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Aldrich) solution, pH 7.4, at 37℃ for 20 min. Tissues were fixed in 10% PBS-buffered formalin overnight at

2–8℃. Hearts were sectioned transaxially, and size of MI was evaluated as the ratio of the sectional area of infarcted tissue of the left ventricle to the sectional area of the whole left ventricle and expressed as a percentage. Both sides of each TTC-stained tissue slice were photographed with a digital camera.

9. Histology and determination of fibrosis area

Rats were killed at various time points after implantation, and their hearts were excised. Hearts were perfusion-fixed with 10% (vol/vol) neutral buffered formaldehyde for 24 hr, transversely sectioned into four comparably thick sections, and embedded in paraffin by routine methods. Sections of 5-μm thickness were mounted on gelatin-coated glass slides to ensure different stains could be used on successive sections of tissue cut through the implantation area. After deparaffinization and rehydration, the sections were stained with hematoxylin and eosin to assess cytologic details such as nuclei, cytoplasm, and connective tissue. Additionally, fibrosis was analyzed by Masson's trichrome staining. The interstitial fibrotic area was measured with MetaMorph software version 4.6 (Universal Imaging Corp.) from Sham group (n=9), MSC group (n=9), and PI3K-C2α group (n=9) and expressed as a percentage of the total left ventricle. Tissue showing inflammatory cell inflammation was sectioned and stained with hematoxylin-eosin and the number of inflammatory cells was counted in five randomly selected fields and carefully analyzed by three people

blinded to the treatment.

10. Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay

The TUNEL Assay was performed according to the instructions of the manufacturer (Chemicon International Inc.). In brief, the excised heart tissues were fixed in 3.7% buffered formaldehyde and embedded in paraffin. Tissue sections, 5-μm thick, were deparaffinized, rehydrated, and rinsed with PBS. A positive control sample was prepared from a normal heart section by treating the section with DNase I (10 U/ml, 10 min at room temperature). The sections were pretreated with 3.0% H_2O_2, subjected to a reaction with the TdT enzyme at 37℃ for 1 hr, and then incubated with digoxigenin-conjugated nucleotide substrate at 37℃ for 30 min. Nuclei exhibiting DNA fragmentation were visualized after staining with 3,3-diamino benzidine (DAB) (Vector Laboratories) for 5 min. The nuclei of apoptotic cardiomyocytes stained dark brown. Lastly, the sections were counterstained with methyl green and then cover-slipped. The sections were observed by light microscopy. Six slices per group were prepared, and 10 different regions per slice were observed.

11. Determination of capillary density

Histological analysis was performed according to the manufacturer's

instructions (R.T.U VECTASTAIN Universal Quick Kit; Vector Laboratories). In brief, excised heart tissues were fixed in 3.7% buffered formaldehyde and embedded in paraffin. Tissue sections, 5-μm thick, were deparaffinized, rehydrated, and rinsed with PBS. Antigen retrieval was performed with 10 mM sodium citrate (pH 6.0) in a microwave for 10 min. Sections were incubated in 3% H_2O_2 to quench endogenous peroxidase activity. Samples were blocked in 2.5% normal horse serum, and then incubated with anti-CD31 antibody. Finally, tissue slides were biotinylated with a pan-specific universal secondary antibody. For quantification, capillaries were counted in 10 randomly chosen fields from two separate slides using the (× 200) objective. The mean number of capillaries per field in the infarcted myocardium was used for statistical analysis.

12. Statistical analysis

Data are expressed as means ±SEM. The significance of differences between two groups were assessed by Student`s t-test. Comparisons between more than two groups were performed by one-way ANOVA using Bonferroni's correction. A p values less than 0.05 was considered significant.

III. RESULTS

1. PI3K-C2α is essential for MSC survival under hypoxic conditions

To investigate whether PI3K signaling is involved in MSC survival, MSCs were isolated and purified as described previously.[21,22] MSCs retained spindle-shaped fibroblastic morphology, through repeated passages, and their identity was confirmed by immunocytochemistry and fluorescence-activated cell sorting analysis. The cultured MSCs expressed CD71, CD90, CD105, and CD106. They expressed neither the hematopoietic marker CD34 nor CD14 (Fig. 2). The endogenous expression of PI3K in hypoxic MSCs was measured by western blot analysis. Endogenous PI3K-C2α expression in MSCs decreased slowly for 6 hr under hypoxic conditions, and then time-dependently and rapidly decreased (Fig. 3). To determine the effect of PI3K on MSC survival under hypoxic conditions, cells were transfected with lentivirus vector containing PI3K under normoxic conditions for over 12 hr. We evaluated the expression levels of PI3K and determined cell viability after a 12 hr incubation under hypoxic conditions. Gene transduction with PI3K rescued the PI3K expression levels of hypoxic MSCs by about 72% compared with control MSCs (Fig. 4A). The survival rate of hypoxic MSCs was reduced by about 40% compared to MSCs cultured under normoxic conditions. However, PI3K-MSC showed a survival rate only slightly attenuated (~10%) compared to control MSCs (Fig. 4B).

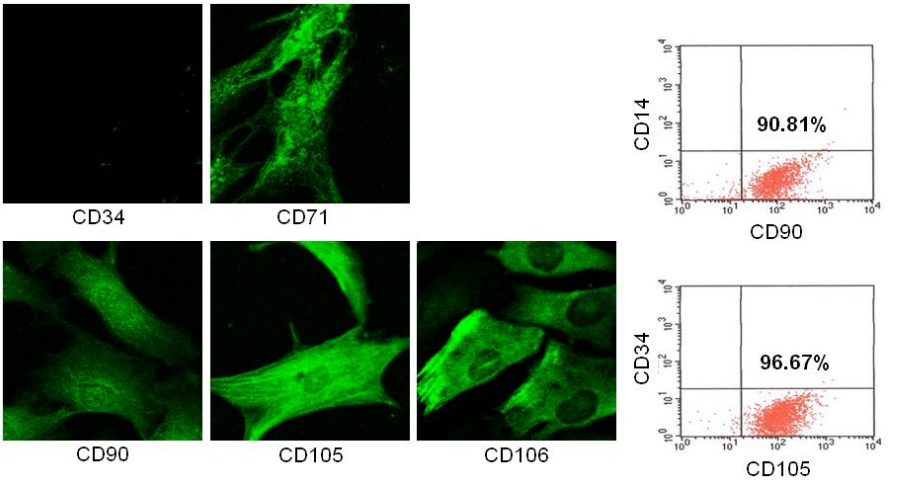

Figure 2. Characterization of isolated MSCs. Most adherent MSCs are practically fibroblastic in morphology, and cells were cultured from bone marrow after density fractionation and are shown at 10 days after plating (Magnification, × 100). MSCs were positive for CD71, CD90, CD105, and CD106 were negative for CD14 and CD34 by immunocytochemistry and flow cytometry.

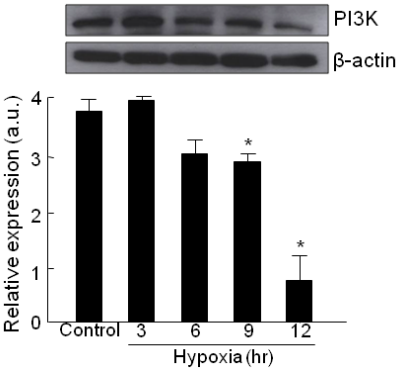

Figure 3. Expression of PI3K-C2α on MSCs under hypoxic condition. At the indicated times, cells were harvested. The expressions of each protein were monitored by SDS-PAGE followed by immunoblot analysis. The band intensities were quantified using the Photo-Image System and each point represented the mean value obtained in 3 experiments. (*p<0.001 vs. Control)

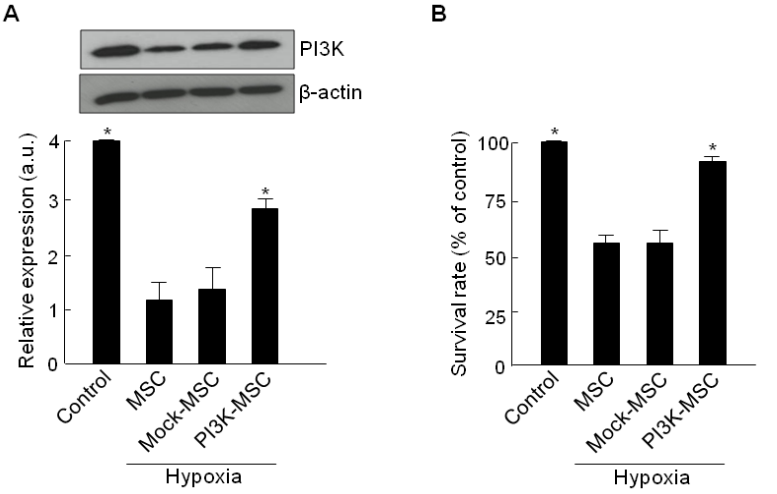

Figure 4. Survival effect by expression of PI3K-C2*α* under hypoxic condition. Figure A shows the results of western blot analysis. PI3K-transduced MSCs were incubated under hypoxic conditions for 12 hr. Figure B shows the results of WST-1 analysis. WST-1 reagent was added to each well and plates were incubated for 2 hr at 37℃. Cell proliferation was measured by spectrophotometry (λ = 570 nm, *p<0.001 vs. hypoxic MSC).

2. The PI3K-C2α-MSCs strengthen the survival signaling under hypoxic condition

As numerous intracellular proteins are activated by cell survival signaling pathways, we assessed the phosphorylation status of certain survival signaling molecules. The phosphorylation of Akt and Bad declined dramatically in hypoxic MSCs compared to normal cells. However, hypoxic PI3K-MSCs showed a 2.6-fold and 2.3-fold increase in levels of phosphorylated Akt and Bad compared to hypoxic MSCs, respectively (Fig. 5A and 5B).

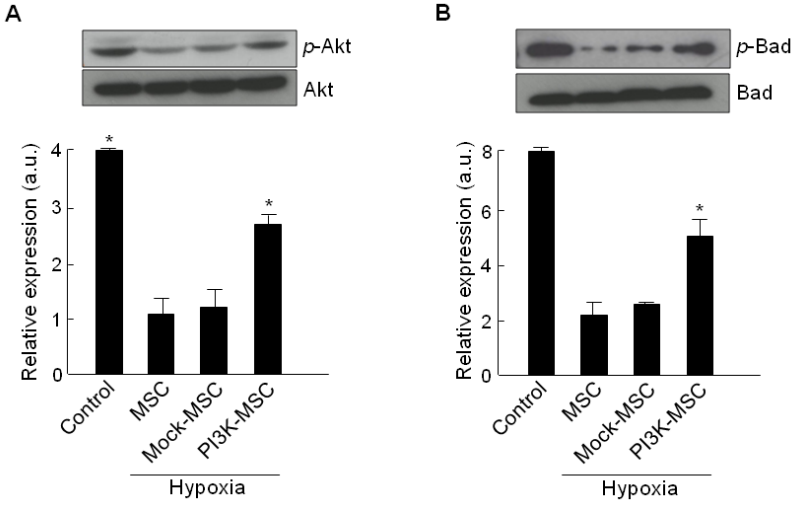

Figure 5. Effect of PI3K on the activity of survival-related proteins. The phosphorylated states or total expressions of each protein were monitored by SDS-PAGE followed by immunoblot analysis with antibodies specific for the Akt, p-Akt, Bad, and p-Bad, respectively. Phosphorylation of Akt (A) and Bad (B) was enhanced in PI3K-MSCs compared to MSCs. The band intensities were quantified using the Photo-Image System and each point represented the mean value obtained in 3 experiments (*p<0.001 vs. hypoxic MSC).

3. Transcription of prosurvival factors is regulated by PI3K-C2α overexpression

It is further examined upregulation of phosphorylated CREB and IKK, which stimulate the transcription of pro-survival factors. Higher levels of phosphorylated CREB and IKK were observed in PI3K-MSCs than hypoxic MSCs under hypoxic conditions (a 1.7-fold and 2.0-fold increase, respectively) (Fig. 6A and 6B).

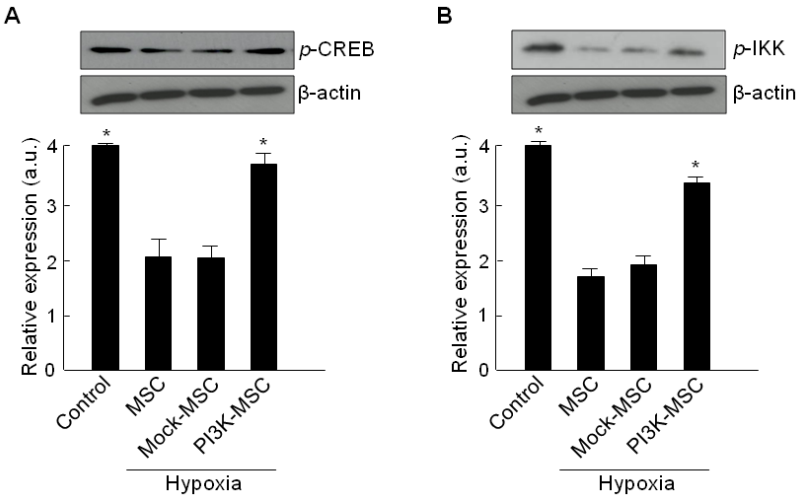

Figure 6. Effects of PI3K on transcription of prosurvival factors of MSCs under hypoxic conditions. The phosphorylated states or total expressions of each protein were monitored by SDS-PAGE followed by immunoblot analysis with antibodies specific for the CREB, p-CREB, IKK, and p-IKK, respectively. Phosphorylation of CREB (A) and IKK (B) was enhanced in PI3K-MSCs compared to MSCs. The band intensities were quantified using the Photo-Image System and each point represented the mean value obtained in 3 experiments (*p<0.001 vs. hypoxic MSC).

4. The PI3K-C2α activation of MSCs reduce the apoptosis-related proteins in hypoxic condition

To investigate the inhibition of the apoptotic pathway in PI3K-MSCs under hypoxic conditions, we first examined PARP expression as a hallmark of apoptosis. Consistent with the decreased cell viability of MSCs, levels of cleaved PARP increased in hypoxic MSCs. However, cleaved PARP levels in hypoxic PI3K-MSCs decreased about 1.8-fold compared to hypoxic MSCs (Fig. 7A). To further examine these effects at a cellular level, the ratio of Bcl-2/Bax was examined. Bcl-2 activity increased by about 2.5-fold after hypoxic stress in PI3K-MSCs compared with hypoxic controls. In contrast, PI3K overexpression significantly reduced Bax activity by 1.7-fold compared to hypoxic MSCs (Fig. 7B). To estimate the numbers of apoptotic and necrotic cells, we analyzed PI single staining in hypoxic MSCs. There were approximately 22.7% less PI-positive PI3K-MSCs than MSCs (Fig. 8).

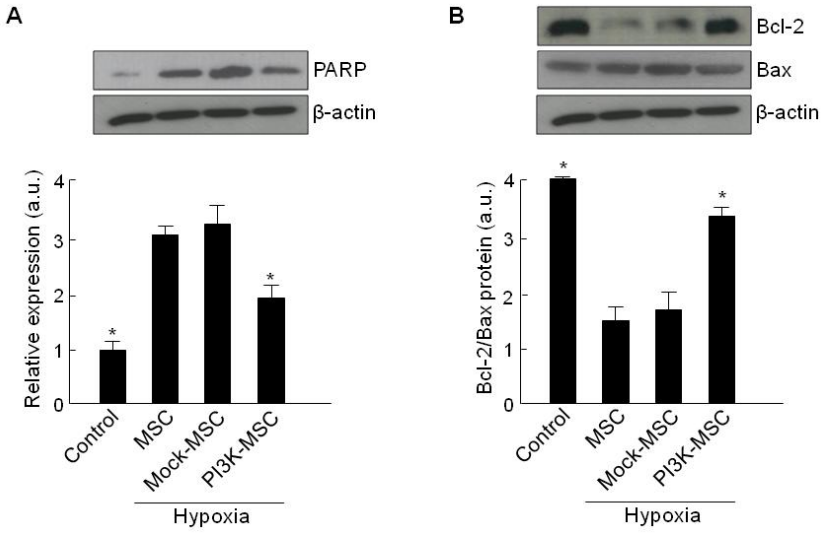

Figure 7. Change in anti-apoptotic signaling on hypoxic MSCs. Expression of PARP, Bcl-2 and Bax was determined by immunoblot analysis in PI3K-C2α-MSCs or MSCs under hypoxic condition. The expressions of each protein were monitored by SDS-PAGE followed by immunoblot analysis with antibodies specific for the PARP, Bcl-2, Bax and β-actin, respectively. Expression of PARP (A), Bcl-2, and Bax (B) was determined by immunoblot analysis in PI3K-MSCs or MSCs after incubation under hypoxic conditions for 12 hr. The band intensities were quantified using the Photo-Image System and each point represented the mean value obtained in 3 experiments (*p<0.01 vs. hypoxic MSC).

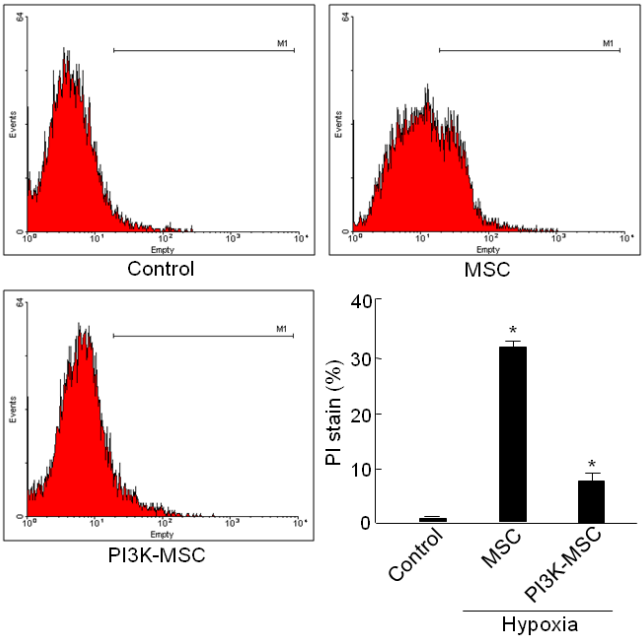

Figure 8. Effect of PI3K on cell death as assessed by PI staining and flow cytometric analysis. Cells were exposed to hypoxic condition for 12 hr. Value is mean±SEM. (*p<0.01 vs. hypoxic MSC).

5. Cell viability is improved in PI3K-C2*α*-MSCs *in vitro* and *in vivo*

To confirm the survival effect of overexpressed PI3K under hypoxic conditions, we performed quantitative in vitro 3D assays. The 3D culture system was derived from cardiac fibroblasts to mimic ischemic heart tissue. The attachment of MSCs was enhanced approximately 1.5-fold by PI3K overexpression compared to hypoxic MSCs (Fig. 9). To further study the effects of PI3K on cell viability in vivo, we transplanted PI3K-MSCs into the border region of the infarcted area. After 3 days, we sacrificed rats with infarcted myocardium and made slide sections with a thickness of about 5-μm. GFP-positive cells in the PI3K-MSCs-injected group were maintained 3-fold longer than GFP-positive cells in the MSC-only group (Fig. 10). These results support our hypothesis that PI3K increases the survival rate and suppresses apoptotic signaling in MSCs under hypoxic conditions.

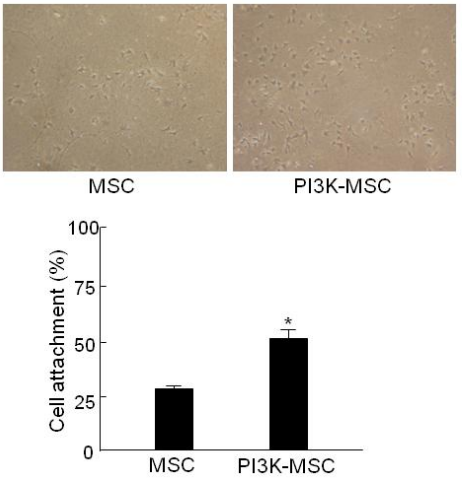

Figure 9. Changes in the survival of PI3K-MSCs. PI3K-MSCs were plated on cardiogel for 3 hr. Data are expressed as the percentage of adhered cell (2×10^4 cells/well), and represented the mean value obtained in 3 experiments done in duplicate (*p<0.01 vs. hypoxic MSC).

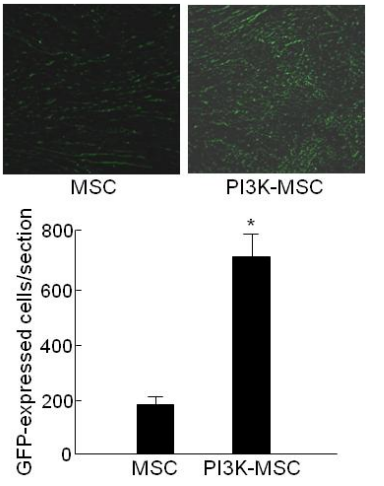

Figure 10. Alteration of stem cell engraftment in ischemic myocardium. Myocardial repair after implantation into infarcted myocardium was analyzed by counting the numbers of GFP-tagged MSCs. Engraftment of PI3K-MSCs was significantly improved at day 3 after injection of cells (1×10^6 cells, $*p<0.01$ vs. MSC-injected rats) compared to engraftment of non-modified MSCs.

6. Intramyocardial injection of PI3K-C2α-MSCs reduces infarct region and enhances ventricular regeneration

To determine the morphological and functional effects of MSCs in infarcted heart tissue, we investigated the survival effects of MSCs in LAD-ligated rat hearts. The effects of PI3K-MSCs on myocardial infarction were evaluated by triphenyl tetrazolium chloride (TTC) and trichrome staining (TRC). We measured infarct size in the left ventricle (LV) using TTC staining. In the sham group, infarct size was 33% of the LV compared to normal rats. Transplantation of MSCs decreased the infarct region by about 19%, while injection of PI3K-MSCs decreased the infarct size by an additional 6% (Fig. 11). There was also significant interstitial fibrosis (21%) in infarcted myocardial compared to normal hearts. In contrast, rats injected with PI3K-MSCs showed a 4% decrease in the interstitial fibrosis area (Fig. 12). A TUNEL assay was used to examine the ratio of apoptotic cells in the transplanted myocardium, because apoptosis plays a major role in cell diminution after myocardial infarction. The number of TUNEL-positive cells was significantly reduced by about 15% in ligated hearts with PI3K-MSCs transplanted compared to the sham group. Compared to the MSC-transplanted group, implantation of PI3K-MSCs also decreased the number of TUNEL-positive cells by about 5% (Fig. 13). Moreover, we observed that rats in the PI3K-MSCs-implanted group had fewer inflammatory cell infiltrates in the border region than the sham and MSC groups (Fig. 14). To

investigate angiogenesis, CD31 staining was used to assess the microvessel density. The total number of vessels was significantly higher in the PI3K-MSC group than in the sham and MSC groups (Fig. 15). These results demonstrate that PI3K-MSCs have reduced apoptosis compared to non-genetically modified MSCs, and regenerated the infarcted myocardial after transplantation to a greater extent than non-genetically modified MSCs.

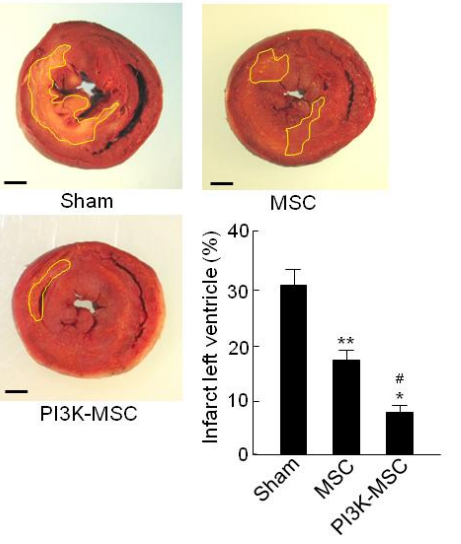

Figure 11. Decrease of infarct size in a PI3K-MSCs-transplanted heart. Intramyocardial injection of MSC reduced left ventricular (LV) infarct size as assessed by TTC staining at 1 week post-MI. Cross-sections showing a larger infarct (yellow) area in the PBS-injected heart. Injection of MSCs reduced the infarcted area, and PI3K-MSCs further decreased the area of necrotic tissue. Each value is the mean±SEM. of six independent experiments ($^{*}p<0.001$, $^{**}p<0.01$ vs. Sham group, $^{\#}p<0.05$ vs. MSC; scale bar=2 mm).

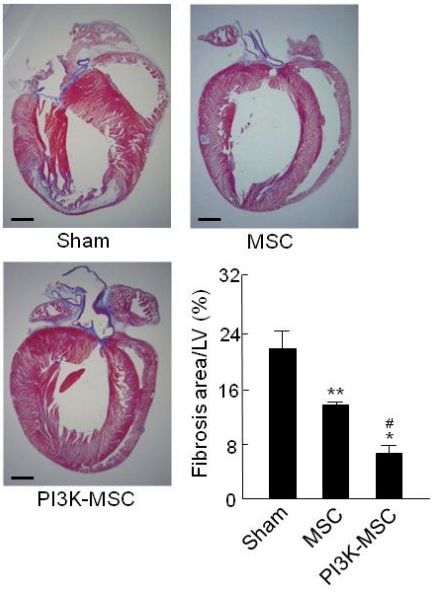

Figure 12. Decline in fibrotic area of left ventricle with transplanted PI3K-MSCs. The LAD of four rat hearts was ligated at the same place. At 1 week after injection of cells, hearts were isolated and cut lengthways. The left panel shows representative Masson's trichrome images from histological sections, and the right histogram shows less fibrosis (blue) in a normal, saline treated-ligated heart, an MSC-transplanted ligated heart, and a PI3K-MSC-transplanted ligated heart ($^{*}p<0.001$, $^{**}p<0.01$ vs. Sham group, $^{\#}p<0.05$ vs. MSC; scale bar=2 mm).

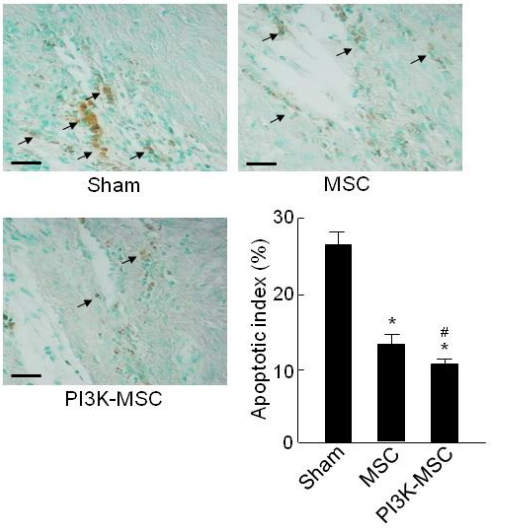

Figure 13. Representative images of TUNEL staining. An apoptosis assay was performed in heart tissue 1 week after reperfusion. Staining for normal nuclei (green) was carried out using methyl green, and apoptotic nuclei were stained brown ($^{*}p<0.01$ vs. Sham group, $^{\#}p<0.05$ vs. MSC; scale bar=100 μm, magnification: ×200).

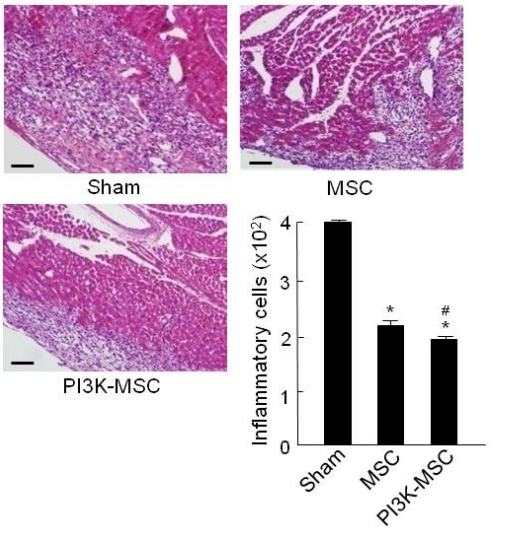

Figure 14. Attenuation of myocardial inflammation in MSC-implanted hearts. H&E staining was performed in MSC-implanted hearts to identify inflammatory cell infiltrate. Each value is the mean ± SEM. of six independent experiments. ($^{*}p<0.01$ vs. Sham group, $^{\#}p<0.05$ vs. MSC; scale bar=40 μm, magnification: ×200)

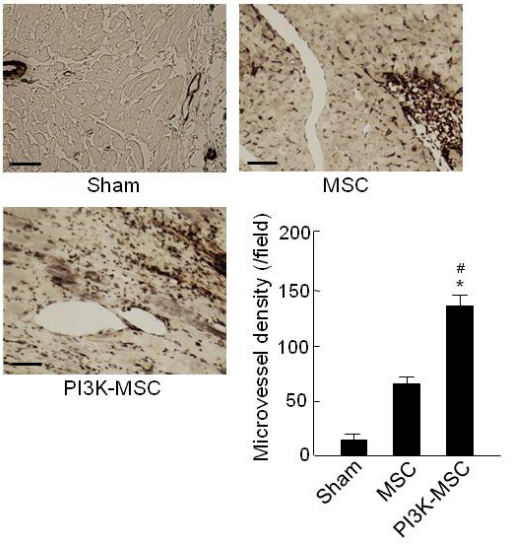

Figure 15. Improvement of microvessel density after PI3K-MSC implantation. The brown spots indicate representative CD31-positive microvessels. Each value is the mean ± SEM. of nine independent experiments ($^{*}p<0.01$ vs. Sham group, $^{\#}p<0.05$ vs. MSC; scale bar=40 μm, magnification: ×200).

IV. DISCUSSION

In recent years, in an attempt to improve the function of ischemic hearts, adult stem cells,[23,24] embryonic stem cells,[25] and various matured[26,27] have been investigated. MSCs in particular are a good resource to enhance myocardial retention[28] and to improve failed cardiac function.[29] Although MSC therapy is a powerful tool for cardiac regeneration, poor survival and low engraftment of transplanted cells in the ischemic heart are major limitations of this strategy.[30] Multiple intracellular signaling processes within MSCs regulate cell attachment, migration, maintenance, and mobilization to ensure stable transplantation of stem cells into infarcted heart tissue. Furthermore, the ischemic microenvironment affects the survival of transplanted cells because of oxygen and nutrient deficiencies. Toma et al. reported that only 0.5% of human MSCs transplanted into an ischemic myocardium survived.[30] Indeed, MSC survival is a crucial issue if they are to be transplanted for cardiac regeneration. To enhance the survival of transplanted MSC in the ischemic heart, we previously studied adhesion-dependent cell survival based on the integrin-mediated molecule, anoikis.[21,22] However, this pathway could not fully account for the poor survival of MSCs after transplantation.

PI3K/Akt signaling regulates several functions critical for survival after transplantation such as cell survival, growth, cycling, and migration. PI3K/Akt

signaling is activated by various growth factors, including insulin growth factor (IGF)-1, epidermal growth factor (EGF), and platelet-derived growth factor (PDGF), and Akt also plays an important role in the cellular function of MSCs.[31,32] Among PI3K isoforms, class II PI3-kinases are linked to different receptor-mediated downstream signaling processes that the other PI3K isoforms. Chemokines and cytokines can induce the overexpression of PI3K-C2α.[33] Previous studies using siRNA sequences have demonstrated that PI3K-C2α plays an important role in controlling cell survival via the intrinsic death pathway; reduction of PI3K-C2α levels below a critical threshold induced apoptotic cell death in a mammalian cell.[34,35]

Ex vivo manipulation of MSCs is very important to improve the cellular survival and transdifferentiation potency of MSCs after implantation. Ex vivo manipulation approaches can be categorized as follows: i) pretreatment with growth factors or cytokines; ii) preconditioning such as hypoxia; and iii) genetic modifications to overexpress anti-death signals.[36] Among these approaches, we investigated the effect of overexpression of PI3K in MSCs on their survival after transplantation.

PI3K overexpression improved the survival of MSCs under hypoxic condition and beneficial effects were also observed in a rat myocardial infarction model. Whereas MSCs showed a strong decrease in expression of endogenous PI3K (Fig. 3), PI3K-MSCs were resistant to apoptosis under hypoxic conditions (Fig.

4). Bad is one of the targets of Akt that is involved in regulation of cell survival. Non-phosphorylation of Bad directly suppresses Bcl-2 and other anti-apoptotic members of the Bcl-2 family; the phosphorylated form of Bad is localized in the cytosol and its proapoptotic ability is thereby neutralized.[37] Our results suggest that phosphorylated Akt and Bad contributes to survival signaling in PI3K-enhanced MSCs (Fig. 5). Furthermore, our results indicate that PI3K induces the production of prosurvival factors in hypoxic MSCs (Fig. 6). In fact, it is known that PI3K-Akt signaling pathway induces the phosphorylation of CREB and IKK, resulting in the transcription of prosurvival regulatory genes.[38] In this study, we assessed changes in the expression of PARP, Bcl-2, and Bax (Fig. 7). The cleavage of poly (ADP-ribose) polymerase (PARP) is a hallmark of apoptosis. The Bax protein, which shows amino acid homology to Bcl-2, induces cell death and can form heterodimers with Bcl-2. When Bax predominates, programmed cell death is accelerated, and the death repressor activity of Bcl-2 is reversed. Therefore, the ratio of Bcl-2 to Bax determines survival or death following an apoptotic stimulus.[39] We examined the numbers of apoptotic and necrotic cells in MSCs and PI3K-MSCs under hypoxic conditions (Fig. 8). PI3K-MSCs were also shown to have enhanced survival in cardiogel (Fig. 9) and the myocardium (Fig. 10), as assessed by counting the number of GFP-positive cells; these results are consistent with a previous study.[22] GFP-positive cells fused with PI3K-MSCs were found in the transplant

region of infarcted hearts. These results indicate that PI3K-MSCs survived for a longer period than the other transplanted stem cells. However, the cellular fate of these transplanted MSCs in the myocardium is not clear. Dzau group found that MSCs overexpressing Akt repaired the ischemic myocardium via paracrine effects, namely the secretion of various cytokines and growth factors.[31] In future studies, we will intend to investigate the fate of PI3K-MSCs and their paracrine effects. Transplantation of PI3K-MSCs into infarcted rat hearts resulted in a dramatic reduction in infarct size and fibrosis area (Figs. 11 and 12). According to our histology results, transplanted PI3K-MSCs decreased the apoptotic index and inflammatory cell infiltration compared to MSCs and controls (Figs. 13 and 14). The inflammatory mechanisms active in MSC-transplanted ischemic myocardium have not been elucidated. Sun et al. reported that implantation of bone marrow cells in ischemic hearts induced myofibroblast repair via the inhibition of neutrophil infiltration and capillary formation.[40] Additionally, the density of microvessels was greater in the PI3K-MSC-transplanted group than the MSC-transplanted group (Fig. 15). Together, our results suggest that genetic modification of MSCs to overexpress PI3K increases MSC survival under hypoxic conditions, and enhances stem cell survival after implantation.

V. CONCLUSION

PI3K is known to stimulate cell proliferation and increase cell viability. As a novel pro-survival strategy to improve MSC survival and to increase the number of viable MSCs in infarcted myocardium, we genetically modified MSCs to overexpress PI3K. Genetic engineering with PI3K enhanced cell survival, viability, and survival signaling, while decreasing apoptotic-regulated signaling in vitro. PI3K-MSCs also had greater cardioprotective effects than MSCs when transplanted into infarcted rat hearts. These findings suggest that overexpression of PI3K in MSCs can improve the function of infarcted hearts.

REFERENCES

1. Frangogiannis NG. The immune system and cardiac repair. Pharmacol Res 2008;58:88–111.
2. Christoforou N, Gearhart JD. Stem cells and their potential in cell-based cardiac therapies. Prog Cardiovasc Dis 2007;49:396–413.
3. Nagaya N, Kangawa K, Itoh T, Iwase T, Murakami S, Miyahara Y, Fujii T, Uematsu M, Ohgushi H, Yamagishi M, Tokudome T, Mori H, Miyatake K, Kitamura S. Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. Circulation 2005;112:1128–1135.
4. Krabbe C, Zimmer J, Meyer M. Neural transdifferentiation of mesenchymal stem cells–a critical review. Apmis 2005;113:831–844.
5. Laflamme MA, Murry CE. Regenerating the heart. Nat Biotechnol 2005;23:845–856.
6. Amado LC, Saliaris AP, Schuleri KH, St John M, Xie JS, Cattaneo S, Durand DJ, Fitton T, Kuang JQ, Stewart G, Lehrke S, Baumgartner WW, Martin BJ, Heldman AW, Hare JM. Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. Proc Natl Acad Sci U S A 2005;102:11474–11479.
7. Chen SL, Fang WW, Ye F, Liu YH, Qian J, Shan SJ, Zhang JJ, Chunhua RZ,

Liao LM, Lin S, Sun JP. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol 2004;94:92–95.

8. Kovacic JC, Graham RM. Stem-cell therapy for myocardial diseases. Lancet 2004;363:1735–1736.

9. Wollert KC, Drexler H. Cell-based therapy for heart failure. Curr Opin Cardiol 2006;21:234–239.

10. Zhang M, Methot D, Poppa V, Fujio Y, Walsh K, Murry CE. Cardiomyocyte grafting for cardiac repair: Graft cell death and anti-death strategies. J Mol Cell Cardiol 2001;33:907–921.

11. Haider H, Ashraf M. Strategies to promote donor cell survival: combining preconditioning approach with stem cell transplantation. J Mol Cell Cardiol 2008;45:554–566

12. Hodgetts SI, Beilharz MW, Scalzo AA, Grounds MD. Why do cultured transplanted myoblasts die in vivo? DNA quantification shows enhanced survival of donor male myoblasts in host mice depleted of CD4+ and CD8+ cells or Nk1.1+ cells. Cell Transplant 2000;9:489–502.

13. ShawRJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature 2006;441:424–430.

14. Amaravadi R, Thompson CB. The survival kinases Akt and Pim as potential pharmacological targets. J Clin Invest. 2005;115:2618–2624

15. Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet. 2006;7:606–619.

16. Bader AG, Kang S, Zhao L, Vogt PK. Oncogenic PI3K deregulates transcription and translation. Nat Rev Cancer. 2005;5:921–929.

17. Wymann MP, Marone R. Phosphoinositide 3-kinase in disease: timing, location, and scaffolding. Curr Opin Cell Biol. 2005;17:141–149.

18. Traer CJ, Foster FM, Abraham SM, Fry MJ. Are class II phosphoinositide 3-kinases potential targets for anticancer therapies? Bull Cancer 2006;93:E53–E58.

19. Wheeler M, Domin J. The N-terminus of phosphoinositide 3-kinase-C2β regulates lipid kinase activity and binding to clathrin. J Cell Physiol. 2006;206:586–593.

20. Meunier FA, Osborne SL, Hammond GR, Cooke FT, Parker PJ, Domin J, Schiavo G. Phosphatidylinositol 3-kinase C2alpha is essential for ATP-dependent priming of neurosecretory granule exocytosis. Mol Biol Cell. 2005;16:4841–4851.

21. Song SW, Chang W, Song BW, Song H, Lim S, Kim HJ, Cha MJ, Choi E, Im SH, Chang BC, Chung N, Jang Y, Hwang KC. Integrin-linked kinase is required in hypoxic mesenchymal stem cells for strengthening cell adhesion to ischemic myocardium. Stem Cells 2009;27:1358–1365.

22. Song H, Chang W, Lim S, Seo HS, Shim CY, Park S, Yoo KJ, Kim BS, Min BH, Lee H, Jang Y, Chung N, Hwang KC. Tissue transglutaminase is essential for integrin-mediated survival of bone marrow-derived mesenchymal stem cells. Stem Cells 2007;25:1431–1438.

23. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med. 2001;7:430–436.

24. Leri A, Kajstura J, Anversa P. Cardiac stem cells and mechanisms of myocardial regeneration. Physiol Rev. 2005;85:1373–1416.

25. van Laake LW, Passier R, Doevendans PA, Mummery CL. Human embryonic stem cell-derived cardiomyocytes and cardiac repair in rodents. Circ Res. 2008;102:1008–1010.

26. Li RK, Mickle DA, Weisel RD, Mohabeer MK, Zhang J, Rao V, Li G, Merante F, Jia ZQ. Natural history of fetal rat cardiomyocytes transplanted into adult rat myocardial scar tissue. Circulation. 1997;96:II-179–II-186.

27. Taylor DA, Atkins BZ, Hungspreugs P, Jones TR, Reedy MC, Hutcheson KA, Glower DD, Kraus WE. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. Nat Med.

1998;4:929–933.

28. Barbash IM, Chouraqui P, Baron J, Feinberg MS, Etzion S, Tessone A, Miller L, Guetta E, Zipori D, Kedes LH, Kloner RA, Leor J. Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. Circulation. 2003;108:863–868.

29. Berry MF, Engler AJ, Woo YJ, Pirolli TJ, Bish LT, Jayasankar V, Morine KJ, Gardner TJ, Discher DE, Sweeney HL. Mesenchymal stem cell injection after myocardial infarction improves myocardial compliance. Am J Physiol Heart Circ Physiol. 2006;290:H2196–H2203.

30. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation. 20021;105:93–98.

31. Noiseux N, Gnecchi M, Lopez-Ilasaca M, Zhang L, Solomon SD, Deb A, Dzau VJ, Pratt RE. Mesenchymal stem cells overexpressing Akt dramatically repair infarcted myocardium and improve cardiac function despite infrequent cellular fusion or differentiation. Mol Ther. 2006;14:840–850

32. Wu CJ, O'Rourke DM, Feng GS, Johnson GR, Wang Q, Greene MI. The tyrosine phosphatase SHP-2 is required for mediating phosphatidylinositol 3-kinase/Akt activation by growth factors. Oncogene. 2001;20:6018–6025.

33. Turner SJ, Domin J, Waterfield MD, Ward SG, Westwick J. The CC chemokine monocyte chemotactic peptide-1 activates both the class I p85/p110 phosphatidylinositol 3-kinase and the class II PI3K-C2alpha. J Biol Chem. 1998;273:25987–25995.

34. Elis W, Triantafellow E, Wolters NM, Sian KR, Caponigro G, Borawski J, Gaither LA, Murphy LO, Finan PM, Mackeigan JP. Down-regulation of class II phosphoinositide 3-kinase alpha expression below a critical threshold induces apoptotic cell death. Mol Cancer Res. 2008;6:614–623.

35. Kang S, Song J, Kang J, Kang H, Lee D, Lee Y, Park D. Suppression of the alpha-isoform of class II phosphoinositide 3-kinase gene expression leads to apoptotic cell death. Biochem Biophys Res Commun. 2005;329:6–10.

36. Song H, Song BW, Cha MJ, Choi IG, Hwang KC. Modification of mesenchymal stem cells for cardiac regeneration. Expert Opin Biol Ther. 2010;10:309–319.

37. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 1997;91:231–241.

38. Deng J, Han Y, Yan C, Tian X, Tao J, Kang J, Li S. Overexpressing cellular repressor of E1A-stimulated genes protects mesenchymal stem cells against hypoxia- and serum deprivation-induced apoptosis by activation of PI3K/Akt. Apoptosis. 2010;15:463–473.

39. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev. 1999;13:1899–1911.

40. Sun J, Li SH, Liu SM, Wu J, Weisel RD, Zhuo YF, Yau TM, Li RK, Fazel SS. Improvement in cardiac function after bone marrow cell therapy is associated with an increase in myocardial inflammation. Am J Physiol Heart Circ Physiol. 2009;296:H43–H50.

ABSTRACT (in Korean)

경색된 심근의 세포 이식에서 phosphoinositide 3-kinase 에 의한 줄기세포 생존율 향상

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은 영 민

심근 재생을 위한 중간엽 줄기세포 치료는 허혈성 심장에서 세포 이식 후 줄기세포 낮은 생존율의 단점이 존재한다. Phosphoinositide 3-kinase (PI3K) 의 멤버는 증식, 성장, 주화성, 그리고 생존과 같은 중요한 세포 반응을 조절한다. 경색된 심장에서 세포 생존을 개선시킬 목적으로, class II PI3K α (PI3K-C2α)를 과 발현 시키기 위해 중간엽 줄기세포에 유전적 조작을 실시하였다. 내재성 PI3K-C2α의 발현은 저산소 상태에서 시간 의존적으로 감소됐다. PI3K-C2α의

과발현은 중간엽 줄기세포에서의 내재성 PI3K-C2α 발현과 생존능을 증가시켰다. Akt, Bad, cAMP-반응 인자-결합 단백질 (CREB) 그리고 IκB 키나아제(IKK)의 인산화를 포함하는 생존 신호는 PI3K-C2α를 과발현 시킨 중간엽 줄기세포(PI3K-C2α-MSCs) 에서 각각 증가되었다. PI3K-C2α-MSCs의 Bcl-2/Bax 비율은 12시간에 저산소 조건에서 큰 증가를 가져왔다. 그러나 poly (ADP-ribose) 폴리머라아제 (PARP)의 쪼개짐과 PI 염색 정도는 저산소 상태의 PI3K-C2α-MSCs 에서 중간엽 줄기세포보다 감소되었다. 중간엽 줄기세포의 부착은 cardiogel (3차원 매트릭스)에서 PI3K-C2α를 과발현 시킴에 의해 강해졌다. 그룹당 9마리 백서를 심근 허혈 후 $1x10^6$ 세포 (20 μl PBS) 이식하였고, 1주일 후 줄어든 허혈 크기와 섬유화 지역을 PI3K-C2α-MSCs 그룹에서 관찰하였다. 형태 분석에 따르면 TUNEL 양성세포들과 호중구세포들은 감소하였으나, 단위 면적당 평균 미세혈관 수는 단순 중간엽 줄기세포 처리 군보다 PI3K-C2α-MSCs에서 증가하였다. 이러한 결과들은 중간엽 줄기세포에서 PI3K-C2α의 과발현이 세포 생존에 도움을 주며 이의 세포 이식은 심근 재생을 높여줌을 시사한다.

핵심되는 말: 중간엽 줄기세포, PI3K-C2α, 생존, 렌티바이러스, 심근 재생